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## Degradation of 1,2-Dichloroethane by *Ancylobacter aquaticus* and Other Facultative Methylophils

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Cultures of the newly isolated bacterial strains AD20, AD25, and AD27, identified as strains of *Ancylobacter aquaticus*, were capable of growth on 1,2-dichloroethane (DCE) as the sole carbon and energy source. These strains, as well as two other new DCE utilizers, were facultative methylophils and were also able to grow on 2-chloroethanol, chloroacetate, and 2-chloropropionate. In all strains tested, DCE was degraded by initial hydrolytic dehalogenation to 2-chloroethanol, followed by oxidation by a phenazine methosulfate-dependent alcohol dehydrogenase and an NAD-dependent aldehyde dehydrogenase. The resulting chloroacetic acid was converted to glycolate by chloroacetate dehalogenase. The alcohol dehydrogenase was induced during growth on methanol or DCE in strain AD20, but no activity was found during growth on glucose. However, in strain AD25 the enzyme was synthesized to a higher level during growth on glucose than on methanol, and it reached levels of around 2 U/mg of protein in late-exponential-phase cultures growing on glucose. The haloalkane dehalogenase was constitutively produced in all strains tested, but strain AD25 synthesized the enzyme at a level of 30 to 40% of the total cellular protein, which is much higher than that found in other DCE degraders. The nucleotide sequences of the haloalkane dehalogenase (*dhlA*) genes of strains AD20 and AD25 were the same as the sequence of *dhlA* from *Xanthobacter autotrophicus* GJ10 and GJ11. Hybridization experiments showed that the *dhlA* genes of six different DCE utilizers were all located on an 8.3-kb *EcoRI* restriction fragment, indicating that the organisms may have obtained the *dhlA* gene by horizontal gene transmission.

Bacterial degradation of 1,2-dichloroethane (DCE) has until now only been studied in detail with a culture of *Xanthobacter autotrophicus* GJ10 that was isolated on this compound as the sole carbon and energy source (13, 14). The bacterium constitutively produces a hydrolytic haloalkane dehalogenase that converts DCE to 2-chloroethanol. The latter compound is degraded further via chloroacetaldehyde to chloroacetic acid by the subsequent action of two inducible enzymes, a pyrrolo-quinoline quinone-containing alcohol dehydrogenase (11) and an NAD-dependent aldehyde dehydrogenase, yielding chloroacetic acid as the product (11, 13). Chloroacetic acid is dechlorinated by the second dehalogenase found in *X. autotrophicus* GJ10, a 2-haloacid dehalogenase that converts the compound to glycolate (13). The conversion of 2-chloroethanol to glycolate was also found in a *Pseudomonas* sp. by Stucki and Leisinger (33).

Until now, only one example of a haloalkane dehalogenase with high activity for DCE has been found. The enzyme, a monomer of 35 kDa, has been purified (15) and crystallized (27), and the three-dimensional structure has been solved (4). Furthermore, the gene has been cloned in *Escherichia coli* and sequenced (12). There are indications that this gene is localized on the pXAU1 plasmid present in strain GJ10 (34). Other hydrolytic haloalkane dehalogenases have been found in gram-positive nonmethylophilic microorganisms (9, 30, 38). These enzymes act on longer-chain chloroalkanes, have no or very low activity for DCE, and seem to be biochemically completely different from the GJ10 haloalkane dehalogenase.

Organisms capable of utilizing volatile chlorinated compounds for growth can be used for the removal of these

substrates from waste streams such as contaminated exhaust gases. This was successfully achieved in trickling filters with dichloromethane degraders (2, 3) but proved more difficult for the above-mentioned *X. autotrophicus* strain utilizing DCE, because of clogging as a result of slime production and sheet formation (1a).

Since the kinetics of the haloalkane dehalogenase is far from optimal and the characteristics of the organisms are not suitable for certain applications, we decided to study the possibility of obtaining new DCE degraders with different properties. The results described here show that all the strains isolated were facultative methylophils and that three of them belonged to the genus *Ancylobacter*. The strains degraded DCE with identical haloalkane dehalogenases and by the same route, but major differences were found in the contents and the regulation of the enzymes involved.

### MATERIALS AND METHODS

**Bacteria and growth conditions.** The DCE-utilizing gram-negative bacterium *X. autotrophicus* GJ10 was described by Janssen et al. (14). *X. autotrophicus* GJ11 was isolated from sediment of the Rhine River as described previously for strain GJ10 (14), and strain RB8 was a gift from W. Frommer (Bayer AG, Leverkusen, Germany). *Ancylobacter aquaticus* AD20 was isolated from a batch enrichment that was inoculated with brackish water sediment (10% [vol/vol]) taken from the Eems channel near Delfzijl, The Netherlands, and supplemented with 1 mM DCE. After repeated subculturing, a pure culture was obtained by streaking on MMY-agar plates that were incubated with 20  $\mu$ l of DCE in the lid of the petri dish. *Pseudomonas* sp. strain GJ1 is a 2-chloroethanol-utilizing bacterium described previously (14).

*A. aquaticus* AD25 and AD27 were isolated from the same

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sediment, but with 1 mM 2-chloroethylvinylether supplied as the only carbon and energy source. The degradation of 2-chloroethylvinylether by these organisms will be described elsewhere.

The mineral medium (MMY) used in all experiments contained the following per liter: 5.37 g of  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 1.36 g of  $\text{KH}_2\text{PO}_4$ , 0.5 g of  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 5 ml of salt solution (14), and 10 mg of yeast extract (Difco). Batch cultures of strain AD25 were supplemented with 30 mg of yeast extract per liter.

Bacterial cultures were grown in serum flasks that were filled to one-third of their volume and were closed gas tight with Teflon-lined screw caps to prevent evaporation of volatile substrates. Carbon sources were added up to 4 mM, calculated as if the compounds were completely dissolved in the water phase. The cultures were incubated at 30°C with rotary shaking. Growth rates of the strains were determined by measuring the  $A_{450}$ . In the case of strain AD25, which grew in flocks, a reproducible growth rate measurement was possible after destruction of the bacterial flocks by pulling 8 ml of culture fluid 20 times through a syringe (volume, 10 ml) with a needle that had a diameter of 1 mm.

Large amounts of cells of strains AD20 and AD25 were grown aerobically in a 10-liter Braun BioStat E fermentor (pH 7, 30°C) in MMY medium to which an additional 5 ml of salts solution per liter and 30 mg of yeast extract per liter were added. Glucose was used as the carbon source.

**Preparation of crude extracts and enzyme purification.** Cells that were in the late exponential growth phase were harvested by centrifugation (10 min at  $10,000 \times g$ ) and resuspended in 10 mM Tris-sulfate buffer (pH 7.5) that contained 1 mM  $\beta$ -mercaptoethanol and 1 mM EDTA (TEM buffer). Cells were washed once in TEM buffer (10 min at  $10,000 \times g$ ) and sonicated. A crude extract was obtained by centrifugation (30 min at  $40,000 \times g$ ).

Cells grown in 10-liter batch cultures were harvested by continuous centrifugation (Sharples TIP), after which the cells were washed once with TEM buffer (10 min at  $10,000 \times g$ ) and sonicated. To protect enzymes against inactivation, extracts were kept at 0 to 4°C.

Purification of haloalkane dehalogenase was performed as described earlier for *X. autotrophicus* GJ10 (15).

**Enzyme assays.** Haloalkane and chloroacetate dehalogenase activities were measured with a colorimetric assay by monitoring halide release as described before (13). Dehalogenase activities were expressed as micromoles of halide produced per minute per milligram of protein (units per milligram).

Alcohol dehydrogenase activities were measured at 30°C by using an oxygen electrode. Phenazine methosulfate was used as an artificial electron acceptor as described earlier (11). One unit is defined as the activity that catalyzes an oxygen consumption rate of 1  $\mu\text{mol/min}$ .

Acetaldehyde dehydrogenase activities were assayed spectrophotometrically at 30°C by monitoring the reduction of NAD at 334 nm (11). One unit of enzyme activity is defined as the production of 1  $\mu\text{mol}$  of NADH per min.

Protein concentrations were determined with Coomassie brilliant blue and bovine serum albumin as the standard.

**Biochemical characterization.** The molecular masses of denatured dehalogenases were determined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (19). Ovotransferrin (78 kDa), bovine serum albumin (67 kDa), ovalbumin (45 kDa), myoglobin (17 kDa), and cytochrome *c* (12.3 kDa)

were used as reference proteins. The gels were stained with Coomassie brilliant blue.

The molecular masses of native purified dehalogenases were estimated by means of gel filtration by using a Sephadex G100 column (80 by 2 cm) in TEM buffer. Bovine serum albumin, ovalbumin, trypsin inhibitor, and cytochrome *c* were used as marker proteins (10 mg of each). The N-terminal amino acid sequences were analyzed as described earlier (36).

**DNA amplification.** Chromosomal DNA was isolated from strains GJ10, GJ11, AD20, AD27, and RB8 grown on nutrient broth. Strain AD25 was grown on brain heart infusion for this purpose. The method used for DNA isolation was adopted from that of Fulton et al. (5). Further purification of DNA was carried out by CsCl-ethidium bromide equilibrium density gradient centrifugation (28). After digestion of DNA with *Hind*III, 50 ng was used for polymerase chain reaction (PCR) amplification according to the standard method described by Sambrook et al. (28). Tween 20 (0.05% [vol/vol]) replaced gelatin as the stabilizing agent. The DNA oligonucleotides used as primers were manufactured by Eurosequence BV, Groningen, The Netherlands, on an Applied Biosystems 380B DNA synthesizer and had the following 5' to 3' nucleotide sequences: ATGATAAATGCAATTCCG (primer A), ACCGCAACTTCCTGCTTGC (B), CTGACC GAAGCTGAGGCC (C), TTCTGTCTCGGCAAAGTG (D), GAAAGGCGCAGCATACGC (E), and TGCAAGCAGG AAGTTGCG (F). After 35 cycles of amplification with primers A and D, the DNA was electrophoresed on an agarose gel, purified by using a GENE CLEAN kit (Bio 101, Inc., La Jolla, Calif.), and directly used for sequencing by the dideoxy chain termination method of Sanger et al. (29) with T7 polymerase. Reactions were run with the primers A, B, C, D, E, and F.

**Hybridization.** A digoxigenin-labeled DNA probe specific for *dhlA* was obtained by incorporation of digoxigenin-11-dUTP (DIG DNA labeling and detection kit; Boehringer, Mannheim, Germany) during PCR amplification essentially as described by Lion and Haas (20). Primers used were located in the coding region of *dhlA* and were 5'-CAG CAATCTAGAGTATC-3' (sense) and 5'-TTCTGTCTCG GCAAAGTG-3' (antisense). The length of the DNA probe was 894 bp, which was in agreement with the electrophoretic mobility of the fragment on agarose gels.

Total DNA was digested with *Eco*RI, separated on an 0.8% agarose gel, and transferred to a nylon membrane (Nytran NY 13N; Schleicher & Schuell) by capillary blotting. Hybridization and detection of the digoxigenin-labeled probe were done with reagents from Boehringer as described by the manufacturer, except that 1% blocking reagent was used in the hybridization buffer.

**GC and MS.** Capillary gas chromatography (GC) and GC-mass spectrometry (MS) were carried out as described before (13, 35).

**Chemicals.** Chemicals used were checked for purity as stated before (13, 35).

## RESULTS

**Isolation and characterization of strains AD20, AD25, AD27, and GJ11.** From the original enrichment culture with DCE as the carbon source, samples were transferred to flasks with new medium and scored for growth on DCE. After about 1 month, growth was observed in several cultures. Three subcultivations were carried out, and cells were streaked on MMY-agar plates incubated with a DCE atmo-

TABLE 1. Characteristics of strains AD20, AD25, and AD27

Strain	Property														
	Gram reaction <sup>a</sup>	Oxi-dase <sup>a</sup>	Cata-lase <sup>a</sup>	β-Galac-tosi-dase <sup>a</sup>	Fermen-tation <sup>b</sup>	Growth at 37°C <sup>b</sup>	Nitrate reduc-tion <sup>b</sup>	N <sub>2</sub> fixa-tion <sup>b</sup>	Mo-tility <sup>a</sup>	Flag-ella-tion <sup>a</sup>	Growth in MMY <sup>c</sup>	Morphology <sup>a</sup>	Colony morphol-ogy <sup>a</sup>	Pig-menta-tion <sup>a</sup>	
AD20	–	+	+	+	–	–	–	–	–	–	Diffuse	Ring-shaped	Circular, convex	Cream	
AD25	–	+	+	+	–	–	–	–	–	–	Flocks	Ring-shaped	Circular, convex	Cream	
AD27	–	NT <sup>d</sup>	NT	NT	–	NT	–	NT	–	–	Diffuse	Ring-shaped	Circular, convex	Cream	

<sup>a</sup> Cells were grown on brain heart infusion-agar.<sup>b</sup> Cells were grown in MMY supplemented with 4 mM glucose.<sup>c</sup> Cells were grown in MMY supplemented with 4 mM DCE.<sup>d</sup> NT, not tested.

sphere. In this way, a pure culture of strain AD20 was obtained. The method used for the isolation of strain GJ11 from sediment of the Rhine River was the same as described for strain GJ10 (13). Strains AD25 and AD27 were isolated on 2-chloroethylvinylether and were also found capable of growth on DCE.

Although strains AD20, AD25, and AD27 were isolated on different halogenated compounds, there was a remarkable resemblance between the three organisms (Table 1). All were gram-negative, nonflagellated, nonmotile, horseshoe-shaped (Fig. 1), facultatively methylotrophic rods. Electron-microscopic examination of strain AD25 revealed the presence of extracellular fibrous material (Fig. 1B and C) which was not found with strain AD20 (Fig. 1A). The strains were capable of growing on methanol, ethanol, ethylene glycol, 1-propanol (weakly), 1,2-propanediol, acetate, propionate, glycerol, pyruvate, lactate, glucose, and galactose. No growth was found with 1-butanol, 1-pentanol, citrate, mannose, lactose, or toluene. On basis of their peculiar morphology and autotrophic growth with methanol, the strains were

identified as *A. aquaticus*, formerly named *Microcycylus aquaticus* (17, 23, 31). The identification of strain AD25 was confirmed by the National Collection of Industrial and Marine Bacteria, Aberdeen, United Kingdom.

A few previously described members of the genus *Ancylobacter* were able to grow chemolithoautotrophically and fix nitrogen (21, 22). Strains AD20 and AD25 did not grow in nitrogen-free medium in the presence of extra vitamins (13) with glucose or pyruvate as the growth substrates under the conditions tested. Autotrophic growth with an atmosphere containing 10% CO<sub>2</sub>, 60% H<sub>2</sub>, 2% O<sub>2</sub>, and N<sub>2</sub> was not found, although in parallel experiments *X. autotrophicus* GJ10 showed growth. Autotrophic growth with methanol as the sole carbon source, previously found with *A. aquaticus* (21), was also observed with the *A. aquaticus* strains studied here. The strains were not gas vacuolated, as was observed with some other *Ancylobacter* spp. (24, 37). Compared with those of other facultative methylotrophs, the properties of strains AD20, AD25, and AD27 found here gave a 100%

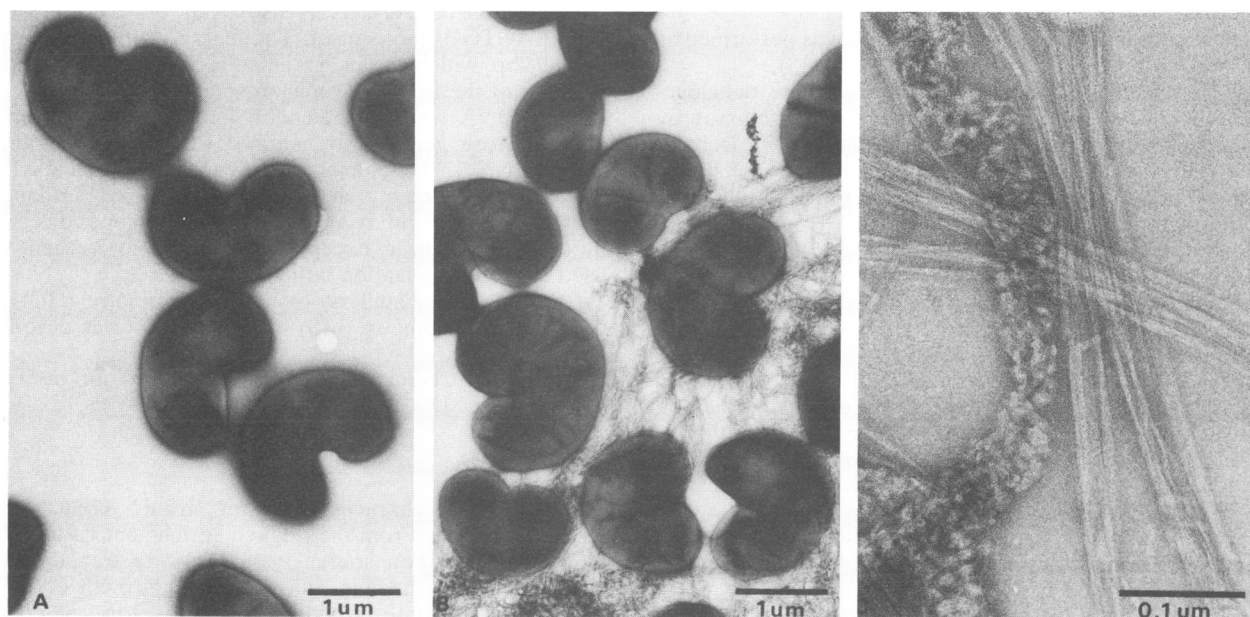


FIG. 1. Electron micrographs of *A. aquaticus* strains AD20 (A) and AD25 (B) and fibrous material of strain AD25 (C). Cells of both strains were grown on DCE. All preparations were stained with 1% uranylacetate.

TABLE 2. Growth rates of strains AD20, AD25, and GJ10 on halogenated aliphatic compounds

Substrate	Growth rate ( $\text{h}^{-1}$ ) of strain		
	AD20 <sup>a</sup>	AD25 <sup>b</sup>	GJ10 <sup>c</sup>
DCE	0.08	0.04	0.11
2-Chloroethanol	0.10	0.07	0.13
Chloroacetic acid	0.07	0.07	— <sup>d</sup>
2-Chloropropionic acid	0.06	0.05	0.08
1-Chloropropane	—	—	0.12
1-Chlorobutane	—	—	0.10

<sup>a</sup> Cells were grown in MMY supplemented with 10 mg of yeast extract per liter.

<sup>b</sup> Cells were grown in MMY supplemented with 30 mg of yeast extract per liter.

<sup>c</sup> Cells were grown in MMY supplemented with 1 ml of vitamins per liter. Data are from reference 13.

<sup>d</sup> —, no growth observed.

score for cluster 3, which represents members of the genus *Ancyllobacter* as described by Green and Bousfield (6).

Strain GJ11 showed the same properties as found earlier with *X. autotrophicus* GJ10 (13), except that strain GJ11 was not able to use toluene as the carbon source and produced more slime than strain GJ10 on nutrient broth-agar plates. It was concluded that strain GJ11 was *X. autotrophicus*. Strain RB8 was found capable of growing on both DCE and methanol but was not further characterized.

**Growth on halogenated compounds.** The utilization of halogenated compounds by strains AD20 and AD25 was determined. Growth tests were done with liquid medium supplemented with a carbon source up to a concentration of 4 mM (Table 2).

Strain AD20 showed relatively fast growth on DCE in MMY (maximum growth rate =  $0.08 \text{ h}^{-1}$ ). However, during growth on DCE, there was an accumulation of up to 0.59 mM 2-chloroethanol in the late exponential phase, which was then degraded to below the detection level ( $<5 \mu\text{M}$ ). Growth of strain AD25 on DCE was exponential, with a maximum growth rate of  $0.04 \text{ h}^{-1}$ , but 2-chloroethanol transiently accumulated up to 2.59 mM. Besides growth on DCE and on 2-chloroethanol, strains AD20 and AD25 were also able to grow on chloroacetic acid and on 2-chloropropionic acid but not on 1-chloropropane, 1-chlorobutane, or 1,3-dichloropropane. 1-Chloro-2-propanol, epichlorohydrin, 2-bromoethanol, and 1,2-dibromopropane were also not growth substrates for strains AD20 and AD25. 2-Bromoethanol ( $10 \mu\text{M}$ ) and 1,2-dibromoethane ( $10 \mu\text{M}$ ) were both very toxic for these strains, and both completely inhibited growth on DCE. This may be due to accumulation of toxic intermediates, most notably bromoacetaldehyde (10).

**Degradation of DCE and intermediates in crude extracts.** Crude extracts prepared from cells grown on DCE were checked for the presence of haloalkane dehalogenase. DCE-dechlorinating activity was found in all organisms. Dehalogenase assays with extracts from cells grown on nonhalogenated compounds indicated the presence of a constitutive haloalkane dehalogenase in strains AD20 and AD25 (Table 3). However, there were significant differences in dehalogenase levels among the various strains. Extracts of the strains AD27, GJ11, and RB8 grown on DCE had haloalkane dehalogenase activities of 936, 203, and 164 mU/mg of protein, respectively. The specific activity in crude extracts of strain AD25 for DCE was 15 to 20 times higher than in crude extracts of *X. autotrophicus* GJ10. This suggested that

TABLE 3. Activities of enzymes of strains AD20, AD25, and GJ10 involved in the conversion of DCE to glycolate

Growth substrate	Enzyme and substrate	Enzyme activity (mU/mg of protein)		
		AD20	AD25	GJ10 <sup>a</sup>
	Haloalkane dehalogenase			
DCE	DCE	541	4,293	232
Glucose	DCE	576	4,247	— <sup>b</sup>
Citrate	DCE	—	—	226
	Alcohol dehydrogenase			
DCE	Methanol	438	212	520
DCE	2-Chloroethanol	487	260	480
DCE	2-Bromoethanol	—	263	—
DCE	Ethanol	394	217	—
Glucose	Methanol	<10	1,970	—
Glucose	2-Chloroethanol	<10	1,821	—
Citrate	2-Chloroethanol	—	—	<10
	Aldehyde dehydrogenase			
DCE	Chloroacetaldehyde	1,387	1,872	109
Glucose	Chloroacetaldehyde	188	417	—
Citrate	Chloroacetaldehyde	—	—	<10
	Chloroacetate dehalogenase			
DCE	Chloroacetate	910	1,035	416
Glucose	Chloroacetate	902	1,486	—
Citrate	Chloroacetate	—	—	462

<sup>a</sup> Data are from references 11 and 13.

<sup>b</sup> —, not determined.

the haloalkane dehalogenase of strain AD25 had a higher activity for DCE or that the organism produced much higher levels of dehalogenase than strain GJ10.

The product of DCE hydrolysis, 2-chloroethanol, was converted further to chloroacetaldehyde by a phenazine methosulfate-linked alcohol dehydrogenase (Table 3). Besides 2-chloroethanol, 2-bromoethanol, methanol, and ethanol were also converted by the alcohol dehydrogenase of strain AD25. This enzyme was induced during growth on DCE in strains GJ10 and AD20 (Table 3). In contrast, strain AD25 possessed a much higher alcohol dehydrogenase activity when cells were grown on glucose than on DCE. Both activities were measured with extracts made from late-exponential-phase cultures. With glucose- or methanol-grown cultures harvested in the mid-exponential phase, alcohol dehydrogenase activities, with methanol as the substrate, of 872 and 420 mU/mg of protein were found. The maximum growth rates of strain AD25 on glucose and methanol were  $0.15$  and  $0.12 \text{ h}^{-1}$ , respectively. Thus, although the growth rate on glucose was higher than that on methanol, strain AD25 produced a higher alcohol dehydrogenase activity during growth on glucose than on methanol. A decrease of the growth rate on glucose during substrate depletion resulted in a higher alcohol dehydrogenase activity.

The accumulation of high concentrations of 2-chloroethanol in the medium during growth on DCE of strain AD25 may have been due to the high activity of the haloalkane dehalogenase compared with the activity of the alcohol dehydrogenase.

In all strains tested, an NAD-dependent aldehyde dehydrogenase that oxidized chloroacetaldehyde was detected (Table 3). The acetaldehyde dehydrogenase activities in strains AD20 and AD25 were also found in cells grown on glucose and were induced to much higher levels during growth on DCE.

The product formed from chloroacetaldehyde, chloroace-

TABLE 4. Purification of haloalkane dehalogenase of *A. aquaticus* AD20

Step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification factor
Crude extract	2,580	755	0.29	100	
Ammonium sulfate	864	260	0.30	35	1
DEAE-cellulose	125	109	0.88	15	3
Hydroxylapatite	32	108	3.46	14	12
Sephadex G100	16	80	5.03	11	17

tic acid, was converted by a constitutive halocarboxylic acid dehalogenase, detected in strains AD20 and AD25 (Table 3). The activities of this enzyme in crude extracts of strains GJ11 and RB8 grown on DCE were 345 and 125 mU/mg of protein, respectively. In crude extracts of strain RB8, grown on glucose, an activity of 55 mU/mg of protein was found, indicating that the chloroacetate dehalogenase was also constitutive in this bacterium.

The results indicated that the pathways of DCE conversion by strains AD20, AD25, AD27, GJ10, GJ11, and RB8 were identical. The regulation of the enzymes involved was different in the various organisms tested, however.

**Purification of haloalkane dehalogenase.** The properties of the purified dehalogenases of strains AD20 and AD25 were studied in more detail to obtain indications of whether the enzymes were identical and to elucidate the dehalogenase content of strains AD20 and AD25. The haloalkane dehalogenase of strain AD20 was purified 17-fold, with a yield of 11% (Table 4), indicating that the dehalogenase of this strain was present at 5 to 6% of the total soluble cellular protein.

The purification factor of the haloalkane dehalogenase of strain AD25 could not be determined because the specific activity of the dehalogenase became 50% reduced during ammonium sulfate precipitation and dialysis. This could have been due to partial denaturation or proteolytic cleavage of the enzyme. The haloalkane dehalogenase activity in crude extracts of strain AD25 grown on glucose in a 10-liter fermentor was 4,633 mU/mg of protein. After purification, a specific activity of 6,267 mU/mg of protein was obtained. SDS-PAGE of the purified dehalogenases of strains AD20, AD25, and GJ10 showed that the enzymes had the same electrophoretic mobility and had an identical molecular mass of 35.0 kDa (Fig. 2). Gel filtration revealed a molecular mass of the native dehalogenase of strain AD20 of 35.5 kDa.

Sequence analysis of the first 34 and 40 N-terminal amino acids of the purified dehalogenases from AD20 and AD25, respectively, revealed no differences. These sequences were identical to the N-terminal sequence of haloalkane dehalogenase of strain GJ10 (12).

**Substrate specificity and products.** The relative activities of the purified dehalogenases for different halogenated compounds were not significantly different for the enzymes from strains AD20, AD25, and GJ10. DCE was converted to 2-chloroethanol. Several new substrates of haloalkane dehalogenase were found, and the products were identified by GC-MS. It was found that the enzyme had activity for 2-bromoethanol, which was converted to ethylene glycol (Table 5). Epichlorohydrin, a widely used chemical (18), was dehalogenated to glycidol (2,3-epoxy-1-propanol). 1,2-Dibromopropane was converted to 1-bromo-2-propanol and to 2-bromo-1-propanol, indicating that the dehalogenases attacked this substrate both at C-1 and C-2. 1,2-Dichloropropane was also converted to the two chloropropanols. The

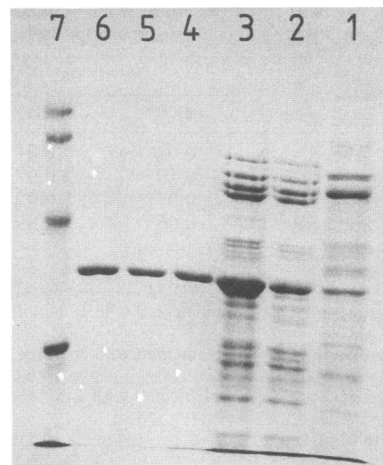


FIG. 2. SDS-PAGE of crude extracts (25  $\mu$ g) of strains GJ10 (lane 1), AD20 (lane 2), and AD25 (lane 3) grown on DCE. Lanes 4 to 6, purified haloalkane dehalogenase (10  $\mu$ g per lane) of strains GJ10, AD20, and AD25, respectively. Lane 7, protein markers.

relative rates of formation of 1-chloro-2-propanol and 2-chloro-1-propanol were 0.26% and 0.34%, respectively, compared with the rate of 1,2-dichloroethane dehalogenation.

**DNA sequence of the haloalkane dehalogenase genes.** To determine whether the haloalkane dehalogenases of several DCE utilizers were identical, the putative *dhlA* genes were amplified by PCR. This was done with purified DNA of strains GJ11, AD20, and AD25. The DNA was digested with *Hind*III before amplification. Electrophoresis on agarose gels revealed that with DNA from all strains, a 0.9-kb DNA fragment was formed by PCR (data not shown). The 0.9-kb DNA fragments were isolated from agarose gels and sequenced. This revealed identical DNA sequences, indicating that the haloalkane dehalogenases of the three strains were the same. The sequences were also identical to the sequence of *dhlA* gene of strain GJ10 (12).

**Southern blotting.** Whether the structural genes of the haloalkane dehalogenase of the DCE utilizers were located on identical or on different restriction fragments was studied.

TABLE 5. Activities of purified haloalkane dehalogenase of strain GJ10

Substrate	Relative activity <sup>a</sup> (%)	Products <sup>b</sup>
DCE	100	2-Chloroethanol
Dichloromethane	9	— <sup>c</sup>
1-Chloropropane	48	—
1,3-Dichloropropane	87	—
1-Chlorobutane	47	1-Butanol
1,4-Dichlorobutane	21	—
Epichlorohydrin	8	Glycidol
1,2-Dibromopropane	133	1-Bromo-2-propanol, 2-bromo-1-propanol
1,2-Dichloropropane	0.6	1-Chloro-2-propanol, 2-chloro-1-propanol
2-Bromoethanol	24	Ethylene glycol

<sup>a</sup> Absolute activity was 6.0 U/mg of protein with DCE as the substrate.

<sup>b</sup> Products were formed with purified haloalkane dehalogenase and identified with GC-MS.

<sup>c</sup> —, not determined.



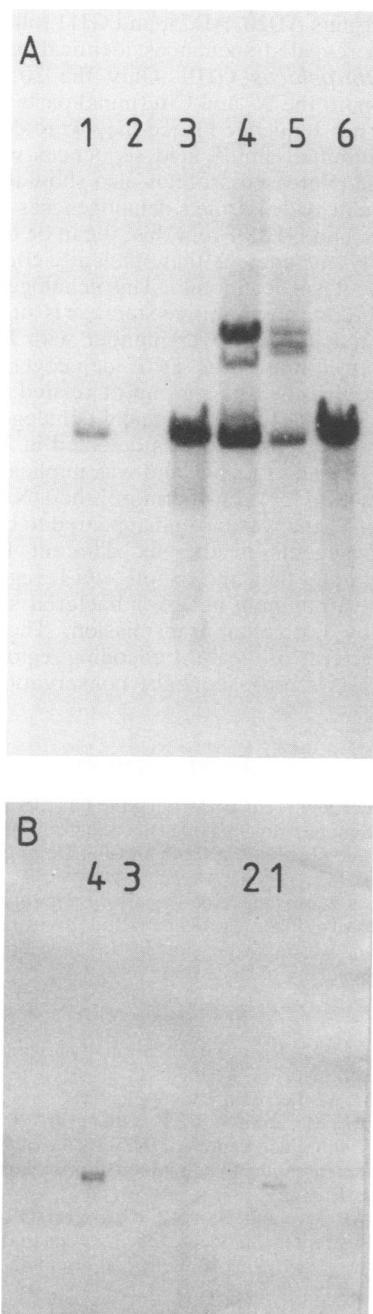


FIG. 3. DNA-DNA hybridization with digoxigenin-labeled probe of the *dhlA* gene of *X. autotrophicus* GJ10 with *Eco*RI-digested total DNA of other DCE degraders. The strains used in each lane were as follows: (A) 1, GJ10; 2, GJ11; 3, AD20; 4, AD25; 5, AD27; 6, RB8; (B) 1, GJ10; 2, GJ11; 3, GJ1; 4, AD27.

To perform this, hybridization experiments were carried out with a *dhlA* gene probe (see Materials and Methods) and *Eco*RI-digested total DNA of strains GJ10, GJ11, AD20, AD25, AD27, and RB8 (Fig. 3). In all cases, an 8.3-kb fragment hybridized with the probe, indicating that the *dhlA* gene of each strain was located on this restriction fragment. No hybridization was found with the DNA of the 2-chloroethanol利用者 strain GJ1, which is in agreement with the

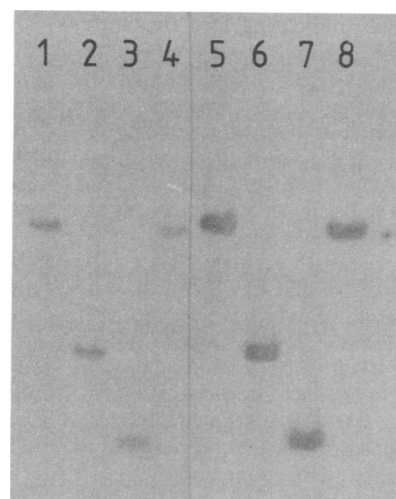


FIG. 4. DNA-DNA hybridization with digoxigenin-labeled probe of the *dhlA* gene of strain GJ10 with digested DNA of strain GJ10 (lanes 1 to 4) and digested DNA of strain AD25 (lanes 5 to 8). Restriction enzymes used were *Eco*RI (lanes 1 and 5), *Bam*HI (lanes 2 and 6), *Hind*III (lanes 3 and 7), and *Pst*I (lanes 4 and 8).

observation that this organism is not capable of dehalogenating DCE (14).

In the case of strain AD25, the Southern blot experiment showed two major bands (Fig. 3). This was caused by partial digestion of the DNA, because in later experiments in which the DNA of the strains AD25 and GJ10 was digested by three different restriction enzymes, single hybridization bands were found (Fig. 4). It was found that the *dhlA* genes of strains AD25 and GJ10 were located on *Bam*HI, *Eco*RI, *Hind*III, and *Pst*I fragments of the same size, indicating that large regions of DNA around *dhlA* were the same or very similar in these organisms.

## DISCUSSION

Three closely related bacterial strains, strains AD20, AD25, and AD27, were found capable of growing on DCE as the only carbon and energy source. The organisms were classified as *A. aquaticus* strains. The ability to grow on xenobiotic compounds has not previously been reported for *Ancylobacter* species (24). Recently, we also isolated a strain of *A. aquaticus* that grows on 2-chloro-1-propanol, indicating that the capacity to degrade halogenated aliphatic compounds may not be unusual among members of this species.

A reason for the fact that the five DCE utilizors studied here, including strain RB8, were facultative methylotrophs might be the involvement of the pyrrolo-quinoline quinone-dependent methanol dehydrogenase in the metabolism of 2-chloroethanol. The enzyme is generally found in methylotrophs (1) and responsible for both methanol (1) and 2-chloroethanol oxidation in strain GJ10 (11). On the other hand, 2-chloroethanol-utilizing organisms that do not grow on methanol have been reported (14, 32, 33).

The synthesis of the alcohol dehydrogenase was repressed in strains GJ10 and AD20 when grown on citrate and glucose, respectively, and induced during growth on methanol and DCE (11). This type of regulation is often found in methylotrophic bacteria (1). In contrast, high levels of alcohol dehydrogenase were synthesized by strain AD25 grown

ing on glucose, indicating that the activity is constitutive. Moreover, this activity was strongly increased in late-exponential-phase cultures grown on glucose. An increase in alcohol dehydrogenase activity during a decreasing growth rate has frequently been observed with different methylotrophs (7, 8, 25, 26). However, cultures of strain AD25 had a higher maximum growth rate with glucose than with methanol as the growth substrate but still possessed in the late exponential phase of growth on glucose a higher methanol dehydrogenase activity. This type of regulation of alcohol dehydrogenase synthesis has not previously been reported for facultative methylotrophic bacteria.

The haloalkane dehalogenase activity, which is rather low in strain GJ10, is 2- to 3-fold higher in strain AD20 and approximately 15- to 20-fold higher in strain AD25. The dehalogenase content of strain GJ10 is 2 to 3% (15), indicating that the dehalogenase content in strain AD25 was at least 30 to 40%, which was confirmed by SDS-PAGE. Such a high level of dehalogenating enzyme is not uncommon for bacteria that utilize chlorinated compounds as growth substrates. Levels of 20 to 30% of dehalogenating enzyme have previously been described for a *Methylobacterium* sp. that degraded dichloromethane (16) and for *Arthrobacter* sp. strain AD2, which grew on epichlorohydrin (35, 36). These enzymes, however, were highly inducible, while the haloalkane dehalogenase of strain AD25 was constitutively produced. The enzyme was very stably present in strain AD25 as well in strain AD20, since after approximately 20 times of being subcultured on brain heart infusion agar plates these organisms were still capable of growing on DCE.

The high dehalogenase content of strain AD25 could be one of the reasons for the unusual regulation of alcohol dehydrogenase synthesis in this organism. Dehalogenases can potentially produce large amounts of intermediate alcohols, as was indeed observed for 2-chloroethanol which was excreted into the medium. In the presence of high levels of an alcohol dehydrogenase, this could lead to toxic concentrations of aldehydes. Chloroacetaldehyde has been implicated as a critical intermediate during DCE metabolism (11). Thus, it is very possible that the observed repression of alcohol dehydrogenase and increased chloroacetaldehyde dehydrogenase synthesis are required to prevent accumulation of toxic aldehydes in the cell. Regulation thus takes place at the level of alcohol dehydrogenase and aldehyde dehydrogenase rather than haloalkane dehalogenase.

The absence of a regulatory system for dehalogenase synthesis in the DCE degraders studied so far could point to a recent evolutionary origin of a DCE catabolic pathway. Regulation of gene expression by the availability of DCE would require a second protein, apart from the enzyme, that recognizes the compound and that influences gene expression. Since DCE did, to our knowledge, not occur in nature before it was introduced by humans, it may be that the period for evolution of a DCE catabolic pathway is too short for generating an efficient regulatory mechanism.

Several new substrates for haloalkane dehalogenase were identified. The dihalogenated compound 1,2-dibromopropane was converted by the haloalkane dehalogenase in two different ways, leading to both 1-bromo-2-propanol and 2-bromo-1-propanol. The corresponding conversion of 1,2-dichloropropane was also found with purified dehalogenase of strain GJ10, although the rate of dechlorination of this important environmental pollutant was very low (36 mU/mg of pure protein).

The sequences of the *dhlA* genes from the various DCE degraders were compared. PCR amplification of the *dhlA*

genes from strains AD20, AD25, and GJ11 followed by DNA amplification revealed sequences identical to that of *dhlA* from *X. autotrophicus* GJ10. Only the 20-bp sequences corresponding to the N- and C-terminal parts of the dehalogenase were not sequenced since they were encoded by the primers. N-terminal amino acid sequences were identical, however. The expressed proteins also show the same properties, as found with purified dehalogenases of the strains AD20, AD25, and GJ10. From this, it can be concluded that until now only one enzyme that efficiently converts DCE to 2-chloroethanol has been found. This dehalogenase seems to be in an evolutionarily primitive state, as is suggested by the observation that the turnover number with DCE is rather low (15). Furthermore, the DCE dehalogenase does not seem to have diverged to a group of related sequences, as occurred with 2-chloropropionic acid dehalogenases (36a).

Recently, a large plasmid was detected in *X. autotrophicus* strain GJ10, and this plasmid was implicated in dehalogenase production (34). Furthermore, the DNA fragment on which the *dhlA* gene was located appeared to be on the same restriction fragments in the six different DCE utilizers tested, indicating that the whole *dhlA* region might be derived from a common ancestor bacterial strain and was transferred by horizontal transmission. The similarity in restriction patterns of the *dhlA* encoding regions of different strains suggests strong sequence conservation during this process.

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